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<b>(21) International Application Number:</b> PCT/IT93/00089 <b>(22) International Filing Date:</b> 10 August 1993 (10.08.93)  <b>(30) Priority data:</b> RM92000603 13 August 1992 (13.08.92) IT  <b>(71) Applicants (for all designated States except US):</b> ISTITUTO SUPERIORE DI SANITA' [IT/IT]; Viale Regina Elena, 299, I-00161 Roma (IT). UNIVERSITA' DEGLI STUDI DI ROMA "LA SAPIENZA" [IT/IT]; P.le Aldo Moro, 5, I-00185 Roma (IT).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> LA ROSA, Giuseppe [IT/IT]; GOMEZ MORALES, Maria Angeles [ES/IT]; POZIO, Edoardo [IT/IT]; Istituto Superiore di Sanità, Viale Regina Elena, 299, I-00161 Roma (IT). CRISANTI, Andrea [IT/IT]; MÜLLER, Hans, Michael [DE/IT]; RANUCCI, Lorella [IT/IT]; RECKMANN, Ingeborg [DE/IT]; Università degli Studi di Roma "La Sapienza", P.le Aldo Moro, 5, I-00185 Roma (IT). COLYZZI, Mario [IT/IT]; Università degli Studio di Roma "La Sapienza", P.le Aldo Moro, 5, I-00185 Roma (IT).		<b>(74) Agents:</b> BANCHETTI, Marina et al.; Ing. Barzanò & Zanardo Roma S.p.A., Via Piemonte, 26, I-00187 Roma (IT).  <b>(81) Designated States:</b> AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> NUCLEOTIDE SEQUENCES CODING FOR CRYPTOSPORIDIUM PROTEINS, POLYPEPTIDES CODED BY SAID SEQUENCES AND KITS FOR THE USE THEREOF		
<b>(57) Abstract</b>  Nucleic acids and polypeptides coded by said nucleic acids, derived from protozoan parasites of <i>Cryptosporidium</i> genus are described. Nucleic acids and peptides are advantageously used for developing detection assays of <i>Cryptosporidium</i> in biological samples of human and animal origin and/or in the environment.		

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NUCLEOTIDE SEQUENCES CODING FOR CRYPTOSPORIDIUM PROTEINS,  
POLYPEPTIDES CODED BY SAID SEQUENCES AND KITS FOR THE USE THEREOF

DESCRIPTION

The invention concerns nucleic acids and  
5 polypeptides coded by said nucleic acids, derived from  
protozoan parasites of *Cryptosporidium* genus. Nucleic  
acids and peptides are advantageously used for  
developing detection assays of *Cryptosporidium* in  
biological samples of human and animal origin and/or in  
10 the environment.

*Cryptosporidium* parasites infect the intestinal  
tract of several animal species. Over the last decade  
the number of infections in humans has dramatically  
increased. Most of the affected patients show a marked  
15 immunodeficiency, with a high incidence of AIDS. The  
immunocompromised patients develop a severe and  
frequently irreversible diarrhoea which causes  
malnutrition and represents a major factor leading to  
death.

20 The prophylaxis of *Cryptosporidium* infections  
is hampered by the lack of reliable immunoassays for  
the detection of the parasite. Moreover, by microscopic  
examination, parasite oocyst are difficult to  
discriminate from several microorganisms that are  
25 morphologically similar to *Cryptosporidium*, like  
*Candida* species.

The development of immunological and molecular  
diagnostic assays highly specific for *Cryptosporidium*  
absolutely requires the biochemical characterisation  
30 and localization of parasite antigens and the cloning  
of corresponding genes. The availability of diagnostic  
assays would permit to develop prophylactic measures  
for immunodeficient patients by detecting the parasites

in the environment (food, water) and in individuals (relatives, nurses) that may act as carriers.

Moreover, no effective therapeutic compounds against *Cryptosporidium* infection are available; therefore there is the need to develop reagents able to prevent the onset of the infection in immunodeficient patients.

The authors of the present invention have obtained a rabbit antiserum raised against a whole lysate of *Cryptosporidium* oocysts. The serum has been used to screen a genomic library of *Cryptosporidium* from infected intestinal mucosal cells, into the expression vector  $\lambda$ gt11. Among clones isolated, clone cpRL3 has been shown to comprise a 2359 bp (SEQ ID No.1) insert with an open reading frame coding for a polypeptide of 786 amino acids (SEQ ID No.2). Scanning of the 67.0 version of the "GENE BANK" data base using the cpRL3 sequence failed to reveal any similarity with known DNA sequences.

The cpRL3 insert has been subcloned into an expression plasmid and the corresponding recombinant polypeptide is produced in *E. coli*, fused at the N-terminus to a stretch of six histidines. The histidine sequence allows a fast and efficient purification by nickel chelate chromatography of the polypeptide encoded by cpRL3. The recombinant polypeptide is used as immunogen in Balb/c mice for producing antisera and monoclonal antibodies. The recombinant protein is highly immunogenic. This sequence codes for a portion of a protein of the oocyst wall of *Cryptosporidium* that has an apparent molecular weight of 190,000 Dalton, named "Cryptosporidium Oocyst Wall Protein" (COWP).

Nucleotide and polypeptide sequences derived from the isolated sequence are advantageously utilized for diagnosis of *Cryptosporidium* infections in patients

and/or animals and for analysis of environmental contamination by *Cryptosporidium* oocysts. Treatment of *Cryptosporidium* infection may be obtained by administration of antibodies raised against COWP.

5           The invention concerns also: - designing of specific oligonucleotides, as primers in polymerase chain reactions (PCR) reactions for the detection of the COWP DNA sequence; - the utilisation of COWP amino acid sequence or fragments thereof to raise antisera and/or monoclonal antibodies for the development of  
10 immunological assays to detect the presence of *Cryptosporidium* and/or COWP molecule; - the use of COWP derived polypeptides, either synthetic or recombinant, as components of diagnostic kits for the  
15 detection of COWP released by parasites; - the utilisation of COWP-derived polypeptides, either synthetic or recombinant, for producing antisera and/or monoclonal antibodies to be employed in the therapy of *Cryptosporidiosis*.

20           It is a specific object of the present invention a polypeptide in a substantially purified form comprising a contiguous sequence coded by a *Cryptosporidium* gene, said gene comprising a nucleotide sequence at least 50 % homologous to the sequence of  
25 SEQ ID No.1. Preferably said contiguous sequence comprises an antigenic determinant of *Cryptosporidium*. More preferably said contiguous sequence is coded by the sequence of SEQ ID No.1, or parts thereof; most preferably said contiguous sequence is comprised in the  
30 aminoacid sequence of SEQ ID No.2.

          It is another object of the invention a diagnostic kit for the detection of *Cryptosporidium* in biological and environmental samples comprising, as  
35 specif ligand, the polypeptide according to the invention.

It is another object of the invention the use of a polypeptide according to the invention for raising antibodies able to detect *Cryptosporidium* infection in biological and environmental samples.

5 It is another object of the invention an antibody obtained using as immunogen a polypeptide according to the invention.

10 It is another object of the invention a diagnostic kit for the detection of *Cryptosporidium* in biological and environmental samples comprising, as specif ligand, an antibody able to react with at least one polypeptide according to the invention.

15 It is another object of the invention an oligonucleotide derived from a *Cryptosporidium* gene, said gene comprising a sequence at least 50 % homologous to the sequence of SEQ ID No.1. Preferably said oligonucleotide has a sequence comprised in the sequence of SEQ ID No.1, or in the complementary sequence of SEQ ID No.1.

20 It is another object of the invention a diagnostic kit for the detection of *Cryptosporidium* in biological and environmental samples comprising, as specif ligand, the oligonucleotide according to the invention.

25 It is another object of the invention a PCR kit for the amplification of *Cryptosporidium* DNA comprising, as specif primer, at least one oligonucleotide according to the invention. Preferably said PCR kit comprises two oligonucleotides having  
30 nucleotide sequences according to the invention.

The invention will be illustrated in the following examples, by making reference to the following figures, wherein:

35 - Figure 1 shows an immunoblot analysis of the mouse serum (M10/01) (A) and of a control mouse serum anti TRAP (B) against: the expression product of the

control plasmid pDS56/RBSII-E-6his-TRAP purified on nickel column (1); the expression product of the plasmid pDS56/RBSII-E-6his-cpRL3 purified on nickel column (2); a protein lysate of *E. coli* cells transformed with pDS56/RBSII-E-6his-cpRL3 induced with IPTG (3); and non induced (4); molecular weight standard are indicated.

- Figure 2 shows an immunoblot analysis using the mouse serum M10/01 (A) and the MAbl IB2 (B) against a protein lysate of *C. parvum* oocysts.

- Figure 3A shows an electrophoresis of PCR products amplified from *C. parvum* DNA (1; 4) and from DNA of the plasmid pDS56/RBSII-E-6his-cpRL3 (2;5) using primers combinations Cry-3/Cry-6 (1;2) or Cry-5/Cry-6 (3;4;5;). As control, PCR reaction without template DNA (3). Figure 3B shows an electrophoresis of PCR products amplified from DNA of several parasite species using primer combination Cry-3/Cry-6: DNA extracted from: *C. parvum* (1), *Sarcocystis* sp. (2), *Giardia lamblia* (3) and *P. falciparum* (4). Figure 3C shows an electrophoresis of PCR products amplified from DNA of progressively diluted *C. parvum* oocysts using the primer combinations Cry-3/Cry-6: molecular standards (1), 160 oocysts (2), 80 oocysts (3), 40 oocysts (4), 20 oocysts (5) 10 oocysts (6). As control, PCR reaction is done without template DNA (7) or in the presence of *P. falciparum* DNA (8). In panels A, B and C, lanes 6, 5 and 1 respectively, DNA markers are 3611; 1166; 606; 517; 396; 318; 263 bp.

Example 1  $\lambda$ gt11 library with DNA extracted from *C. parvum* infected calf intestinal mucosa

To develop a *C. parvum* genomic expression library, DNA extracted from the intestinal mucosa of an infected calf is used.

A newborn calf is infected with  $6 \times 10^8$  oocysts of *C. parvum* MI ISS-1 (Pozio et al. 1992.

Trans. R. Soc. Trop. Med. Hyg. 86:636-638). After 5 days, the gut is opened, cut into segments of 30 cm each and washed in phosphate-buffered saline (PBS). Nitro-cellulose filters (soaked in PBS) of the same size as the gut segments are applied to the mucosal side for a few seconds. Filters are progressively numbered and processed for DNA extraction. A small sample is removed from each filter and analysed by microscopy to determine whether parasites have been removed. The sample from each filter is incubated with 1% glutaraldehyde in cacodylate buffer for 2 h. The filters are dehydrated through an increasing ethanol series, embedded in Epon, and cured at 60°C for 24 h. Sections are cut at 0.2- $\mu$ m thickness and stained with toluidine blue. The analysis reveals that nitro-cellulose filters remove only the superficial layer of mucosal cells, together with a large number of parasites.

Only DNA extracted from filters that have removed a large number of parasites are used. DNA is digested with EcoRI and cloned in  $\lambda$ gt11 EcoRI digested, 3' end to the coding sequence of  $\beta$ -galactosidase gene. Phage DNA with cloned inserts is packaged *in vitro* (Boehringer Mannheim *in vitro* packaging kit) to generate the library. The quality of the library is evaluated by analysing the sizes of a subset of inserts by polymerase chain reaction (PCR) with oligonucleotides corresponding to the flanking sequences of the EcoRI site of the  $\beta$ -galactosidase gene. The library has a complexity of  $4.5 \times 10^6$  plaques and an estimated average insert size of 1,800 bp.

The expression library is analysed by use of a rabbit serum developed against purified oocysts of *C. parvum* MI ISS-1. The serum is used after removal of the background reactivity by several absorptions on filters soaked with bacterial and phage lysates. Specific

antibodies bound to filters are detected by use of a second anti-rabbit antibody conjugated to alkaline phosphatase. An insert, named cpRL3, consisting of a 2,359-bp open reading frame encoding a polypeptide of 786 amino acids, is isolated. The lack of both a start codon and a stop codon indicates that the sequence represents part of the coding sequence of the isolated parasitic gene.

Example 2 Expression of the cpRL3 sequence in *E. coli*

The DNA insert cpRL3 is cloned in the EcoRI site of plasmid pDS56/RBSII-E<sup>-</sup> 6xHis (a pDS56/RBSII derived plasmid containing an EcoRI site in the polylinker). The expression unit of this vector is under the control of an isopropyl- $\beta$ -thiogalactopyranoside (IPTG)-inducible promoter and yields a fusion between a stretch of 6 histidines and the amino terminus of the inserted sequences (Stuber et al. 1990, Eur. J. Immunol 20:819-824). cpRL3 is expressed in *E. coli* M15 carrying the lac repressor-producing plasmid pUHA1. Induction is performed in LB medium for 4 h at 37°C; 1 mM IPTG is added when the cell density reaches an optical density at 600 nm of 0.6.

Example 3 Purification of recombinant polypeptide 6xHis-cpRL3

The expression product of the cpRL3 sequence (recombinant polypeptide 6xHis-cpRL3) is purified in a single-step procedure, by nickel chelate affinity chromatography (Stuber et al., *ibid.*). In brief, one litre of an induced culture of M15(pUHA1) cells carrying plasmid pDS56/RBSII-E-6xHis-cpRL3 is harvested and stirred for 3 h in 100 ml of 6 M guanidine hydrochloride, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 8. The suspension is centrifuged at 10,000 x g, and the supernatant is directly applied to a nickel column (NTA-resin, Diagen). After an equilibration step with 8 M urea, 100

mM  $\text{NaH}_2\text{PO}_4$ , 10 mM Tris, pH 8, 6xHis-cpRL3 is eluted by lowering the pH of the urea solution stepwise to pH 4. From one litre of culture, 1 mg of 6xHis-cpRL3 is obtained.

5     Example 4 Immunoblotting

Parasite lysates are obtained from *Cryptosporidium* oocysts purified by Percoll gradient centrifugation (Waldman, E., et al. 1986. J. Clin. Microbiol. 23:199-200). Parasites are lysed by  
10 incubation of pellet of  $2 \times 10^7$  oocysts with 0.2 ml of sample buffer (33 mM Tris-HCl, pH 6.8, 190 mM glycerol, 0.1% SDS). Bacterial lysates are obtained by treatment of  $10^9$  induced or non induced *E. coli* cells with 1 ml of sample buffer. Proteins in total cell lysates are  
15 separated by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, U.K. 1970. Nature (London) 227:680-685) and electroblotted onto nitro-cellulose filters (blotting buffer, 25 mM Tris, 192 mM glycine, 20% methanol). Non-specific adsorption of antibodies to the  
20 nitro-cellulose is prevented by saturation of the filters with 1% bovine serum albumin in 2x TBST (20 mM Tris-HCl, pH 8, 300 mM NaCl, 0.1% Tween 20) for 2 h at room temperature. Nitrocellulose filters are incubated with antibodies for 2 h at room temperature. After  
25 extensive washing with 2x TBST, antibodies bound to the filters are detected by use of goat anti-mouse immunoglobulin (heavy and light chains) conjugated to alkaline phosphates (Promega). Phosphatase activity is disclosed by incubation of the filters with 0.3 mg of  
30 Nitro Blue Tetrazolium and 0.15 mg of 5-bromo 4-chloro-3-indolyl phosphate per ml in 100 mM Tris-HCl (pH 9.5)-100 mM NaCl-5 mM  $\text{MgCl}_2$ .

Example 5 Monoclonal antibody production

After purification by nickel chelate  
35 chromatography, recombinant polypeptide 6xHis-cpRL3 is used as immunogen to develop specific antisera and

monoclonal antibodies. BALB/c mice are immunised three times with 50  $\mu$ g of purified 6xHis-cpRL3 polypeptide in complete (for the first immunisation) or incomplete Freund's adjuvant. Five days after the last  
5 immunisation, mouse spleen cells are fused with x63 Ag 8653 myeloma cells and subsequently screened for antibody production (Kohler, G. and C. Milstein 1975 Nature (London) 256:495-497).

The supernatants of cultures from growing  
10 hybrids are tested in an enzyme-linked immunosorbent assay (ELISA) against 6xHisCpRL3. Immunised mice develop an antibody titer of 1/500,000. Figure 1 (A and B) shows the ability of one of the antisera to recognise specifically by immunoblot the recombinant  
15 polypeptide encoded by cpRL3. The antiserum recognises the recombinant polypeptide in a total lysate of bacteria in which the expression of the cpRL3 insert is induced with IPTG (Fig. 1A, lane 3). The antiserum also recognize the polypeptide after purification by the  
20 nickel chelate chromatography. The specificity of the reaction is demonstrated by the lack of reactivity against *E. coli* proteins, as well as against an unrelated recombinant protein, TRAP, expressed from the control plasmid pDS56/RBSII-6xHis(TRAP), also sharing  
25 the six histidine amino-terminal tail. Antibodies developed against the expression product of insert cpRL3 (mouse antiserum and monoclonal antibody 2B11) detect, in the lysate of *Cryptosporidium* oocysts, a protein of the apparent molecular weight of 190,000  
30 Dalton, (Figure 2 A and B). These results indicates that the expression product of the insert cpRL3 is part of a *Cryptosporidium* protein expressed in the parasite oocysts.

#### Example 6 Immunofluorescence microscopy

35 Purified parasite oocysts are air dried on a coverslip and fixed in cold acetone for 5 min. Non-

specific binding is prevented by pre-incubation of the samples in PBS containing 1% bovine albumin. Primary antibodies (culture supernatant) are allowed to react for 40 min at room temperature, and the secondary fluorescinated antibody (Becton Dickinson goat anti-mouse) is allowed to react for 20 min. Observation of the samples is carried out with confocal microscopic apparatus (Bio-Rad Laboratories).

The protein is localised by immunofluorescence using the monoclonal antibody 2B11 on a preparation of oocysts fixed with acetone. The monoclonal 2B11 specifically binds to a protein of oocyst wall of *Cryptosporidium*. The result of the immunolocalization is confirmed by confocal immunofluorescence analysis which shows that the reactivity is detected on the surface of the oocyst wall. On the basis of these observations, the *Cryptosporidium* protein encoded by the insert cpRL3 is named as *Cryptosporidium* oocyst wall protein, COWP.

By immunofluorescence, monoclonal antibodies can identify as few as 100 oocysts/ml of stools. The detection limit of this method is determined resuspending known numbers of oocysts in samples of non infected stools.

#### Example 7 ELISA assays

Monoclonal antibodies produced against the product of the insert cpRL3 are able to recognise COWP in solution when used in ELISA assays based on antigen capture with two antibodies. Therefore specific antibodies directed against this protein or fragments thereof having an antigenic activity, as the product of cpRL3, can be employed to develop diagnostic assay to reveal *Cryptosporidium* infection.

#### Example 8 PCR

For all PCR experiments, the cpRL3 sequence is amplified in a standard 50  $\mu$ l PCR reaction mixture

(Saiki, R K., et al. 1988. Science 239:487-491; Scharf, S. J. et al. 1986. Science 233:1076-1078) for 35 cycles at 94,5°C (1 min), 58°C (30 sec); 72°C(1 min), with a Lab Line thermal cycler. The final concentration of MgCl<sub>2</sub> is 2 mM. Primers used are:  
Cry3 (5'GTCCTACTGGATTCACTCTAC-3')  
coding strand, nt.722-742 of SEQ ID No. 1;  
Cry5 (5'-CCAGGACATCATCATGGTCATTCTCATGGGC-3')  
coding strand, nt. 1099-1129 of SEQ ID No. 1;  
Cry6 (5'-CCGAATATGTAACACATTTATCCGC-3')  
non coding strand, nt. 1828-1852 of complementary strand of SEQ ID No. 1.

For amplification of the cpRL3 sequence from *Cryptosporidium* oocysts, samples are incubated for 5 min under reducing conditions and boiled for 10 min thereafter. TaqI polymerase is purchased from Perkin-Elmer Co.

The ability of two oligonucleotide combinations to amplify by PCR the sequence of cpRL3 is shown in Figure 3A. Both of oligonucleotide combinations Cry3/Cry6 and Cry5/Cry6 are able to amplify DNA segments of the expected molecular weight from the plasmid pDS56/RBSII-E-6xHis (cpRL3) and from DNA of *Cryptosporidium*. The amplification reaction using oligonucleotides Cry3/Cry6 is highly specific, (figure 3B). Oligonucleotides in fact do not amplify any DNA segment when DNA of other protozoa (*Giardia lamblia*, *Plasmodium falciparum* o *Sarcocystis suis hominis*) is used as template. In PCR experiments the oligonucleotide combination Cry3/Cry6 is able to amplify a specific DNA segment from as few as 40 oocysts of *Cryptosporidium*, (Figure 3C). These data indicate that by using combination of oligonucleotides corresponding different regions of cpRL3 sequence, PCR can be employed to detect the presence of *Cryptosporidium*.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Nucleotide sequences coding for Cryptosporidium proteins, polypeptides coded by said sequences and kits

(iii) NUMBER OF SEQUENCES: 2

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2359 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

(A) ORGANISM: Cryptosporidium parvum

## (ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 1..2359

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAA TTC GAA TGC CCA CCA GGT ACA ATT TTA AAA GAT GAT CAA TGT CAA	48
Glu Phe Glu Cys Pro Pro Gly Thr Ile Leu Lys Asp Asp Gln Cys Gln	
1 5 10 15	
TCG ATA GAA AGA GTT GAT ACA ATT TGT CCA CCA GGG TTT GTA GAT AAT	96
Ser Ile Glu Arg Val Asp Thr Ile Cys Pro Pro Gly Phe Val Asp Asn	
20 25 30	
GGC GAA GAT TGT GTC CAA TTT TCT GCA CCA GAG AAA ATT TGC CCC CAA	144
Gly Glu Asp Cys Val Gln Phe Ser Ala Pro Glu Lys Ile Cys Pro Gln	
35 40 45	
GGA TTT TCT CTT TCC GGA AAA CAA TGT GTT AAA ACA GAA TCT GCT CCA	192
Gly Phe Ser Leu Ser Gly Lys Gln Cys Val Lys Thr Glu Ser Ala Pro	
50 55 60	
AGA TTA ACA GAA TGC CCA CCA GGT ACA ACC TTG GAA AAT AAC AGT TGT	240
Arg Leu Thr Glu Cys Pro Pro Gly Thr Thr Leu Glu Asn Asn Ser Cys	
65 70 75 80	
ATT TCA TAT GAA CTA GAA GAT GCC ATT TGT CCA CCT GGA TAT CTC GAC	288
Ile Ser Tyr Glu Leu Glu Asp Ala Ile Cys Pro Pro Gly Tyr Leu Asp	
85 90 95	
AAT GGA TCA GAC TGT GTT CAG TTT TCT CAA CCA GAA AAG GAG TGT CCA	336
Asn Gly Ser Asp Cys Val Gln Phe Ser Gln Pro Glu Lys Glu Cys Pro	
100 105 110	
ACA GGT TTT GTA TTA ATT GGA AAA CAA TGT ACC CAA ACT ACT CAA GCT	384
Thr Gly Phe Val Leu Ile Gly Lys Gln Cys Thr Gln Thr Thr Gln Ala	
115 120 125	
CCA CCA CAA CCA GAG TGT CCT CCA GGT ACA AAC CTG GTA AAT GGA CAA	432
Pro Pro Gln Pro Glu Cys Pro Pro Gly Thr Asn Leu Val Asn Gly Gln	
130 135 140	
TGC CAA AAA GTT GAA AGG ATA AAT ATG GTA TGT CCA ACT GGT TTT ATT	480
Cys Gln Lys Val Glu Arg Ile Asn Met Val Cys Pro Thr Gly Phe Ile	
145 150 155 160	
GAT AAT GGT ACA AAT TGT GCT TCT TTC TCC GCA CCA AAC AGA GAA TGC	528
Asp Asn Gly Thr Asn Cys Ala Ser Phe Ser Ala Pro Asn Arg Glu Cys	
165 170 175	
CCA CCT GGA TAT ACA CTT TCT GGA TCC CAA TGC GAG CAA ATA AAA GAA	576
Pro Pro Gly Tyr Thr Leu Ser Gly Ser Gln Cys Glu Gln Ile Lys Glu	
180 185 190	

GCA CCT CCT GTT TCA GAA TGT CCA CCA GGA TAT AAA CTT CAA GGA AAT 624  
 Ala Pro Pro Val Ser Glu Cys Pro Pro Gly Tyr Lys Leu Gln Gly Asn  
 195 200 205

CAA TGT ACT GCA CTA AAA ATG ATC GAT GCT ATC TGC CCA GAT GGA TTT 672  
 Gln Cys Thr Ala Leu Lys Met Ile Asp Ala Ile Cys Pro Asp Gly Phe  
 210 215 220

TTA CCA AAT GGA GAC GAT TGT ATC CAA TTT TCT CCT GCT TCA ACT GTA 720  
 Leu Pro Asn Gly Asp Cys Ile Gln Phe Ser Pro Ala Ser Thr Val  
 225 230 235 240

TGT CCT ACT GGA TTC ACT CTA CAA AAT CAA CAG TGT GTT CAA ACA ACT 768  
 Cys Pro Thr Gly Phe Thr Leu Gln Asn Gln Gln Cys Val Gln Thr Thr  
 245 250 255

ACC TCA CCA AAA ACA CCA GAA TGT CCT CCA GGT TCT GCG TTG GAT GGA 816  
 Thr Ser Pro Lys Thr Pro Glu Cys Pro Pro Gly Ser Ala Leu Asp Gly  
 260 265 270

GAC TCG TGC ACA AGA CTT GTT CCC GGG GCT CTT CAA TAC GTT TGT CCT 864  
 Asp Ser Cys Thr Arg Leu Val Pro Gly Ala Leu Gln Tyr Val Cys Pro  
 275 280 285

GTT GGT ACT AGA GAG GGG GAC GTT TGC GTA GAG AGA TCG ATT AGT TCG 912  
 Val Gly Thr Arg Glu Gly Asp Val Cys Val Glu Arg Ser Ile Ser Ser  
 290 295 300

CCT GTT TTG GAA TGC CCA CCT GGT TAT TCA TTG GAA ACA GGT AAA CAA 960  
 Pro Val Leu Glu Cys Pro Pro Gly Tyr Ser Leu Glu Thr Gly Lys Gln  
 305 310 315 320

TGT GTT AGA AGA AGC CAA TAT GAC TGT TCA GTA ACA ACT TAT GTT ACA 1008  
 Cys Val Arg Arg Ser Gln Tyr Asp Cys Ser Val Thr Thr Tyr Val Thr  
 325 330 335

GAG TGT AAA ACA CCT GAT GTT AAA GCA CTA AGA AGA TTA GCA GCT GCA 1056  
 Glu Cys Lys Thr Pro Asp Val Lys Ala Leu Arg Arg Leu Ala Ala Ala  
 340 345 350

AAA GAA ACA TCA ACA GTT TAT GAA ACA TCT GAG ATA CAA AAT CCA GGA 1104  
 Lys Glu Thr Ser Thr Val Tyr Glu Thr Ser Glu Ile Gln Asn Pro Gly  
 355 360 365

CAT CAT CAT GGT CAT TCT CAT GGG CAT TCA CAT TCA CAA GTT ATA CCA 1152  
 His His His Gly His Ser His Gly His Ser His Ser Gln Val Ile Pro  
 370 375 380

ATT CAA ACC CAG AAT ATA CAT ACA CAA CAT CAT AAA GAG GCT CCA AGG 1200  
 Ile Gln Thr Gln Asn Ile His Thr Gln His His Lys Glu Ala Pro Arg  
 385 390 395 400

CCA	ATT	TGT	GAA	GAT	GTT	CCA	AAA	ATT	ACC	CCA	AAA	ACT	TGT	ACA	AAA	1248
Pro	Ile	Cys	Glu	Asp	Val	Pro	Lys	Ile	Thr	Pro	Lys	Thr	Cys	Thr	Lys	
			405						410						415	
GCT	GAT	TCT	GTC	CCA	GCT	GTG	CCT	ATT	TGC	GAG	AAC	AAT	GCT	GAA	CTT	1296
Ala	Asp	Ser	Val	Pro	Ala	Val	Pro	Ile	Cys	Glu	Asn	Asn	Ala	Glu	Leu	
			420					425					430			
GTA	GGA	AAA	GAA	TGT	GTA	TTA	ACA	AAT	TAC	TAC	CCA	TTA	GAA	GCA	ATT	1344
Val	Gly	Lys	Glu	Cys	Val	Leu	Thr	Asn	Tyr	Tyr	Pro	Leu	Glu	Ala	Ile	
		435					440					445				
TGT	CAA	GAT	GGA	ACA	AGA	TCA	AAA	GAG	TGT	GCT	AAG	TTT	GTA	AAA	ACT	1392
Cys	Gln	Asp	Gly	Thr	Arg	Ser	Lys	Glu	Cys	Ala	Lys	Phe	Val	Lys	Thr	
	450					455					460					
CCA	CCT	ACT	TTA	AAA	TGT	CCG	CCA	GGT	TCT	GTA	GAT	GTA	GGA	TCT	CAA	1440
Pro	Pro	Thr	Leu	Lys	Cys	Pro	Pro	Gly	Ser	Val	Asp	Val	Gly	Ser	Gln	
	465				470					475					480	
TGT	CAA	GTT	AAC	AAA	TAT	TCA	CCA	TAT	GAT	CTT	GCA	TGC	CCT	GCA	GGA	1488
Cys	Gln	Val	Asn	Lys	Tyr	Ser	Pro	Tyr	Asp	Leu	Ala	Cys	Pro	Ala	Gly	
			485						490					495		
TAT	GCA	TTG	GTT	GGA	GAC	AAA	TGC	GCT	ACC	ACA	AGA	GAA	AAA	GTT	TGC	1536
Tyr	Ala	Leu	Val	Gly	Asp	Lys	Cys	Ala	Thr	Thr	Arg	Glu	Lys	Val	Cys	
			500					505					510			
CCG	AAT	GAA	AGT	TGC	CAA	AGA	GTT	GTA	ACT	GCG	CCT	GTT	TCT	TTA	ACT	1584
Pro	Asn	Glu	Ser	Cys	Gln	Arg	Val	Val	Thr	Ala	Pro	Val	Ser	Leu	Thr	
		515					520					525				
TGT	CCC	CCT	GGA	TAT	CAC	CAA	ATA	GAT	GAA	GTT	ATG	AAT	ATT	TCT	GCT	1632
Cys	Pro	Pro	Gly	Tyr	His	Gln	Ile	Asp	Glu	Val	Met	Asn	Ile	Ser	Ala	
	530					535					540					
CAT	CCA	CAC	CAC	AGA	CAC	TTA	GCT	GGG	GTT	CAA	TCT	ACT	TCT	CAA	AAG	1680
His	Pro	His	His	Arg	His	Leu	Ala	Gly	Val	Gln	Ser	Thr	Ser	Gln	Lys	
	545				550					555					560	
GGA	TAT	TCT	CAT	GGA	CAT	AAA	TAT	ACT	CCT	GTA	ATT	TCT	CAG	CCA	CCA	1728
Gly	Tyr	Ser	His	Gly	His	Lys	Tyr	Thr	Pro	Val	Ile	Ser	Gln	Pro	Pro	
			565						570					575		
CAA	CCA	GTT	CCA	GTT	GTT	GCT	CCT	ATT	CAG	CAA	ATG	AAA	TGC	ATC	CAT	1776
Gln	Pro	Val	Pro	Val	Val	Ala	Pro	Ile	Gln	Gln	Met	Lys	Cys	Ile	His	
			580					585					590			
GCA	AAC	CAT	GCT	CCA	TAT	AAT	CTT	ATC	TGT	CCT	GTT	GGA	TCA	AGA	CTT	1824
Ala	Asn	His	Ala	Pro	Tyr	Asn	Leu	Ile	Cys	Pro	Val	Gly	Ser	Arg	Leu	
		595					600					605				

GTA Val	ACG Ala	GAT Asp	AAA Lys	TGT Cys	GTT Val	ACA Thr	TAT Tyr	TCG Ser	GAT Asp	AAA Lys	ATA Ile	TGT Cys	CCA Pro	AAT Asn	GGT Gly	1872
610																
AAT Asn	TGC Cys	GAG Glu	CGT Arg	ATA Ile	TAT Tyr	AAT Asn	GAG Glu	CCT Pro	GCT Ala	GAA Glu	TTA Leu	GTA Val	TGC Cys	CCT Pro	CCA Pro	1920
625 630 635 640																
GGA Gly	TTC Phe	TCA Ser	TCA Ser	TCT Ser	AAA Lys	CCA Pro	ATT Ile	CAG Gln	CCA Pro	ATA Ile	AGC Ser	CAT His	TCT Ser	CAT His	ATT Ile	1968
645 650 655																
AAC Asn	CAT His	CCA Pro	AAT Asn	GTT Val	TCT Ser	GTT Val	CCC Pro	GTC Val	CAA Gln	CCA Pro	CAA Gln	ACT Thr	ATT Ile	AAC Asn	CAA Gln	2016
660 665 670																
CCA Pro	CAA Gln	GTA Val	ATT Ile	CAA Gln	CAA Gln	AGA Arg	CAA Gln	GTA Val	AAT Asn	TAT Tyr	CAG Gln	CCA Pro	CAA Gln	GTA Val	ATT Ile	2064
675 680 685																
CAT His	CAA Gln	ACA Thr	CAG Gln	GAA Glu	ATT Ile	TTA Leu	ACA Thr	ACT Thr	TAT Tyr	CCA Pro	ACT Thr	CCA Pro	GTT Val	TAC Tyr	CAA Gln	2112
690 695 700																
ACC Thr	GGC Gly	ACA Thr	ATT Ile	TAT Tyr	CAA Gln	GGA Gly	CAT His	CAT His	CAT His	CAT His	CAT His	CAT His	CAT His	CAT His	CAC His	2160
705 710 715 720																
AGA Arg	AAT Asn	CTA Leu	GCT Ala	TCC Ser	CCT Pro	GAG Glu	TGC Cys	ATT Ile	AAG Lys	ACA Thr	ATT Ile	TCA Ser	GTA Val	CCT Pro	TAT Tyr	2208
725 730 735																
ATT Ile	TTA Leu	AAA Lys	TGC Cys	GAA Glu	TCT Ser	CCA Pro	TTT Phe	ATT Ile	TTA Leu	GAT Asp	GGC Gly	GAC Asp	AAA Lys	TGT Cys	ATC Ile	2256
740 745 750																
GAA Glu	AAA Lys	ACA Thr	GAA Glu	AAA Lys	ATT Ile	TGT Cys	CTA Leu	CAA Gln	GGT Gly	GAC Asp	TGC Cys	AGA Arg	AAA Lys	CAA Gln	GTC Val	2304
755 760 765																
GTC Val	GTT Val	CCA Pro	CCA Pro	ACT Thr	CTT Leu	TCA Ser	TGT Cys	CCA Pro	CAA Gln	GGT Gly	TAC Tyr	AGA Arg	AAT Asn	GCC Ala	AAC Asn	2352
770 775 780																
GGA Gly	ATT Ile	C														2359
785																

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 786 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Glu	Phe	Glu	Cys	Pro	Pro	Gly	Thr	Ile	Leu	Lys	Asp	Asp	Gln	Cys	Gln	1	5	10	15
Ser	Ile	Glu	Arg	Val	Asp	Thr	Ile	Cys	Pro	Pro	Gly	Phe	Val	Asp	Asn	20	25	30	
Gly	Glu	Asp	Cys	Val	Gln	Phe	Ser	Ala	Pro	Glu	Lys	Ile	Cys	Pro	Gln	35	40	45	
Gly	Phe	Ser	Leu	Ser	Gly	Lys	Gln	Cys	Val	Lys	Thr	Glu	Ser	Ala	Pro	50	55	60	
Arg	Leu	Thr	Glu	Cys	Pro	Pro	Gly	Thr	Thr	Leu	Glu	Asn	Asn	Ser	Cys	65	70	75	80
Ile	Ser	Tyr	Glu	Leu	Glu	Asp	Ala	Ile	Cys	Pro	Pro	Gly	Tyr	Leu	Asp	85	90	95	
Asn	Gly	Ser	Asp	Cys	Val	Gln	Phe	Ser	Gln	Pro	Glu	Lys	Glu	Cys	Pro	100	105	110	
Thr	Gly	Phe	Val	Leu	Ile	Gly	Lys	Gln	Cys	Thr	Gln	Thr	Thr	Gln	Ala	115	120	125	
Pro	Pro	Gln	Pro	Glu	Cys	Pro	Pro	Gly	Thr	Asn	Leu	Val	Asn	Gly	Gln	130	135	140	
Cys	Gln	Lys	Val	Glu	Arg	Ile	Asn	Met	Val	Cys	Pro	Thr	Gly	Phe	Ile	145	150	155	160
Asp	Asn	Gly	Thr	Asn	Cys	Ala	Ser	Phe	Ser	Ala	Pro	Asn	Arg	Glu	Cys	165	170	175	
Pro	Pro	Gly	Tyr	Thr	Leu	Ser	Gly	Ser	Gln	Cys	Glu	Gln	Ile	Lys	Glu	180	185	190	
Ala	Pro	Pro	Val	Ser	Glu	Cys	Pro	Pro	Gly	Tyr	Lys	Leu	Gln	Gly	Asn	195	200	205	

Gln Cys Thr Ala Leu Lys Met Ile Asp Ala Ile Cys Pro Asp Gly Phe  
 210 215 220  
 Leu Pro Asn Gly Asp Asp Cys Ile Gln Phe Ser Pro Ala Ser Thr Val  
 225 230 235 240  
 Cys Pro Thr Gly Phe Thr Leu Gln Asn Gln Gln Cys Val Gln Thr Thr  
 245 250 255  
 Thr Ser Pro Lys Thr Pro Glu Cys Pro Pro Gly Ser Ala Leu Asp Gly  
 260 265 270  
 Asp Ser Cys Thr Arg Leu Val Pro Gly Ala Leu Gln Tyr Val Cys Pro  
 275 280 285  
 Val Gly Thr Arg Glu Gly Asp Val Cys Val Glu Arg Ser Ile Ser Ser  
 290 295 300  
 Pro Val Leu Glu Cys Pro Pro Gly Tyr Ser Leu Glu Thr Gly Lys Gln  
 305 310 315 320  
 Cys Val Arg Arg Ser Gln Tyr Asp Cys Ser Val Thr Thr Tyr Val Thr  
 325 330 335  
 Glu Cys Lys Thr Pro Asp Val Lys Ala Leu Arg Arg Leu Ala Ala Ala  
 340 345 350  
 Lys Glu Thr Ser Thr Val Tyr Glu Thr Ser Glu Ile Gln Asn Pro Gly  
 355 360 365  
 His His His Gly His Ser His Gly His Ser His Ser Gln Val Ile Pro  
 370 375 380  
 Ile Gln Thr Gln Asn Ile His Thr Gln His His Lys Glu Ala Pro Arg  
 385 390 395 400  
 Pro Ile Cys Glu Asp Val Pro Lys Ile Thr Pro Lys Thr Cys Thr Lys  
 405 410 415  
 Ala Asp Ser Val Pro Ala Val Pro Ile Cys Glu Asn Asn Ala Glu Leu  
 420 425 430  
 Val Gly Lys Glu Cys Val Leu Thr Asn Tyr Tyr Pro Leu Glu Ala Ile  
 435 440 445  
 Cys Gln Asp Gly Thr Arg Ser Lys Glu Cys Ala Lys Phe Val Lys Thr  
 450 455 460  
 Pro Pro Thr Leu Lys Cys Pro Pro Gly Ser Val Asp Val Gly Ser Gln  
 465 470 475 480

Cys Gln Val Asn Lys Tyr Ser Pro Tyr Asp Leu Ala Cys Pro Ala Gly  
 485 490 495  
 Tyr Ala Leu Val Gly Asp Lys Cys Ala Thr Thr Arg Glu Lys Val Cys  
 500 505 510  
 Pro Asn Glu Ser Cys Gln Arg Val Val Thr Ala Pro Val Ser Leu Thr  
 515 520 525  
 Cys Pro Pro Gly Tyr His Gln Ile Asp Glu Val Met Asn Ile Ser Ala  
 530 535 540  
 His Pro His His Arg His Leu Ala Gly Val Gln Ser Thr Ser Gln Lys  
 545 550 555 560  
 Gly Tyr Ser His Gly His Lys Tyr Thr Pro Val Ile Ser Gln Pro Pro  
 565 570 575  
 Gln Pro Val Pro Val Val Ala Pro Ile Gln Gln Met Lys Cys Ile His  
 580 585 590  
 Ala Asn His Ala Pro Tyr Asn Leu Ile Cys Pro Val Gly Ser Arg Leu  
 595 600 605  
 Val Ala Asp Lys Cys Val Thr Tyr Ser Asp Lys Ile Cys Pro Asn Gly  
 610 615 620  
 Asn Cys Glu Arg Ile Tyr Asn Glu Pro Ala Glu Leu Val Cys Pro Pro  
 625 630 635 640  
 Gly Phe Ser Ser Ser Lys Pro Ile Gln Pro Ile Ser His Ser His Ile  
 645 650 655  
 Asn His Pro Asn Val Ser Val Pro Val Gln Pro Gln Thr Ile Asn Gln  
 660 665 670  
 Pro Gln Val Ile Gln Gln Arg Gln Val Asn Tyr Gln Pro Gln Val Ile  
 675 680 685  
 His Gln Thr Gln Glu Ile Leu Thr Thr Tyr Pro Thr Pro Val Tyr Gln  
 690 695 700  
 Thr Gly Thr Ile Tyr Gln Gly His His His His His His His His  
 705 710 715 720  
 Arg Asn Leu Ala Ser Pro Glu Cys Ile Lys Thr Ile Ser Val Pro Tyr  
 725 730 735  
 Ile Leu Lys Cys Glu Ser Pro Phe Ile Leu Asp Gly Asp Lys Cys Ile  
 740 745 750

Glu Lys Thr Glu Lys Ile Cys Leu Gln Gly Asp Cys Arg Lys Gln Val  
755 760 765

Val Val Pro Pro Thr Leu Ser Cys Pro Gln Gly Tyr Arg Asn Ala Asn  
770 775 780

Gly Ile  
785

## CLAIMS

1. A polypeptide in a substantially purified form comprising a contiguous sequence coded by a *Cryptosporidium* gene, said gene comprising a nucleotide sequence at least 50 % homologous to the sequence of SEQ ID No.1.
2. A polypeptide according to Claim 1 wherein said contiguous sequence comprises an antigenic determinant of *Cryptosporidium*.
3. A polypeptide according to any of previous claims wherein said contiguous sequence is coded by the sequence of SEQ ID No.1, or parts thereof.
4. A polypeptide according to Claim 3 wherein said contiguous sequence is comprised in the aminoacid sequence of SEQ ID No.2.
5. A diagnostic kit for the detection of *Cryptosporidium* in biological and environmental samples comprising, as specif ligand, the polypeptide according to the invention.
6. Use of a polypeptide according to the invention for raising antibodies able to detect *Cryptosporidium* infection in biological and environmental samples.
7. An antibody obtained using as immunogen a polypeptide according to any of Claims from 1 to 4.
8. A diagnostic kit for the detection of *Cryptosporidium* in biological and environmental samples comprising, as specif ligand, an antibody able to react with at least one polypeptide according to any of Claims from 1 to 4.
9. An oligonucleotide derived from a *Cryptosporidium* gene, said gene comprising a sequence at least 50 % homologous to the sequence of SEQ ID No.1.
10. An oligonucleotide according to Claim 9 having a sequence comprised in the sequence of SEQ ID No.1, or in the complementary strand of SEQ ID No. 1.

11. A diagnostic kit for the detection of *Cryptosporidium* in biological and environmental samples comprising, as specif ligand, the oligonucleotide according to Claim 9 or 10.
- 5 12. A PCR kit for the amplification of *Cryptosporidium* DNA comprising, as specif primer, at least one oligonucleotide according to Claim 9 or 10.
- 10 13. A PCR kit according to Claim 12 comprising, as specif primer, two oligonucleotides according to Claim 9 or 10.

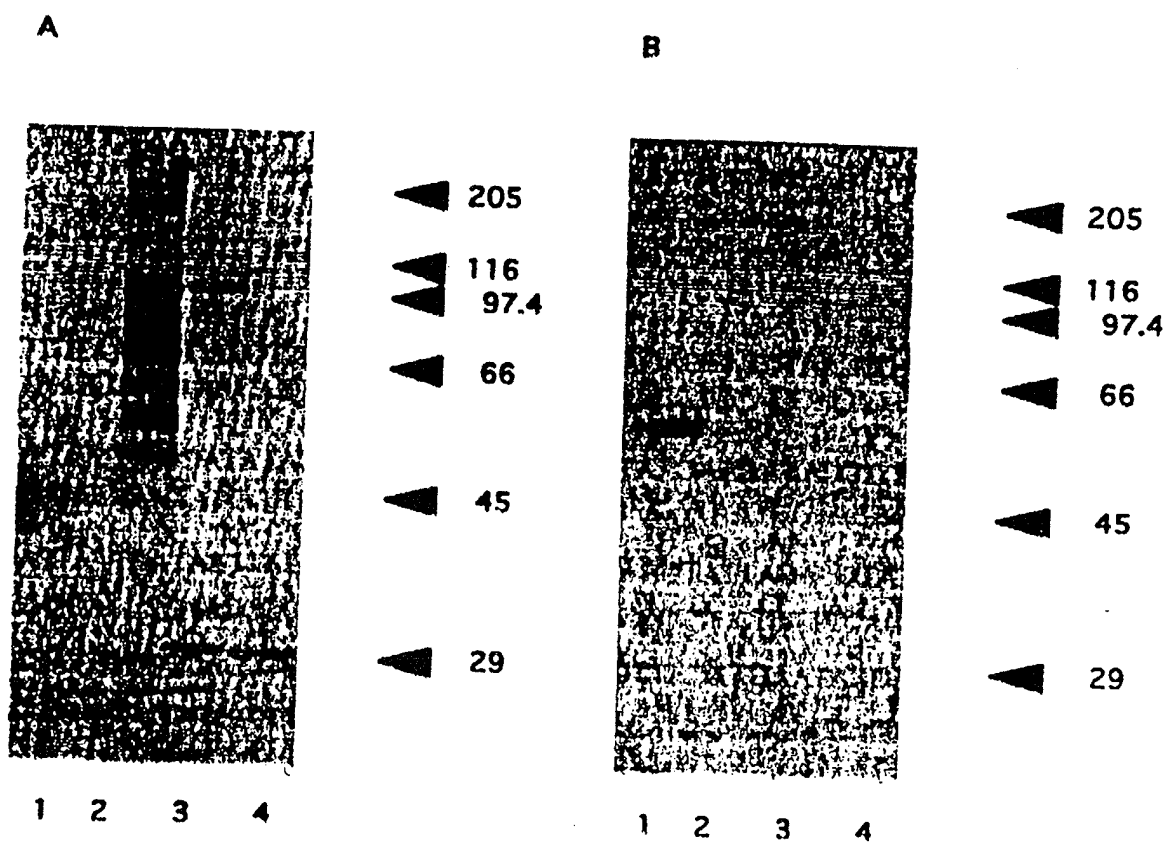


FIG. 1

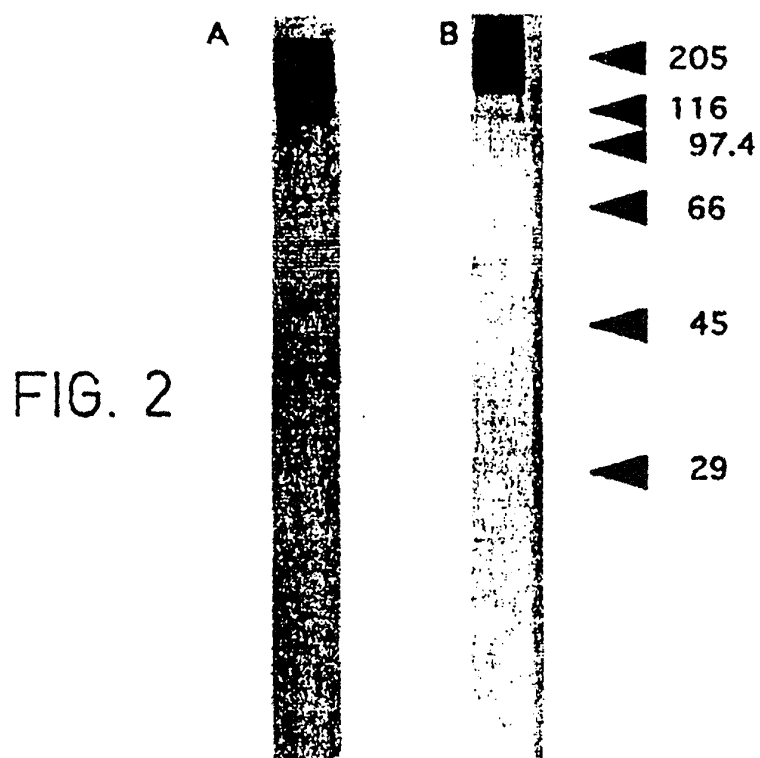
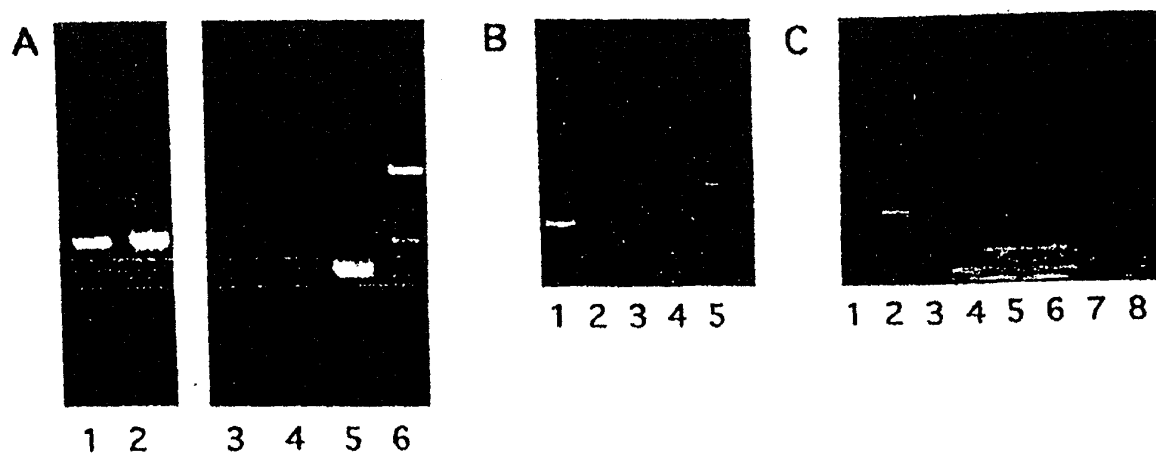


FIG. 3



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/IT 93/00089

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/30 C07K13/00 G01N33/68 C12Q1/68 C07K15/28

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	INFECTION AND IMMUNITY vol. 61, no. 6, June 1993, WASHINGTON US pages 2347 - 2356 RANUCCI, L. ET AL. 'Characterization and immunolocalization of a Cryptosporidium protein containing repeated amino acid motifs' see the whole document see figure 2 ---	1-13
P,X	MOLECULAR AND BIOCHEMICAL PARASITOLOGY vol. 56, no. 1, November 1992 pages 69 - 78 LALLY, N. C. ET AL. 'A 2359-base pair DNA fragment from Cryptosporidium parvum encoding a repetitive oocyst protein' see the whole document see figure 4 ---	1-13
-/--		

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

3 January 1994

Date of mailing of the international search report

04 -02- 1994

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Fax: (+31-70) 340-3016

Authorized officer

Espen, J

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/IT 93/00089

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>J. PROTOZOOL. vol. 38, no. 6 , November 1991 pages 76S - 78S DYKSTRA, C. C. ET AL. 'Construction of genomic libraries of Cryptosporidium parvum and identification of antigen-encoding genes' see table 2 -----</p>	1-13